Increased Smad Expression and Activation Are Associated with Apoptosis in Normal and Malignant Prostate after Castration

Grgor Brodin, Peter ten Dijke, Keiko Funa, Carl-Henrik Heldin, and Marene Landström

Ludwig Institute for Cancer Research, Biomedical Center, SE-751 24 Uppsala [G. B., P. t. D., C-H. H., M. L.], and Institute of Anatomy and Cell Biology, Göteborg University, SE-405 30 Göteborg [K. F.], Sweden

ABSTRACT

Transforming growth factor (TGF-β) is induced in the prostate after castration and has been implicated in apoptosis of epithelial cells during involution. TGF-β1-mediated receptor activation induces phosphorylation of Smad2 and Smad3, which form complexes with Smad4, that translocate to the nucleus to regulate transcription of target genes. Smad6 and Smad7 antagonize the action of signal-transducing Smads.

We have examined the immunohistochemical expression of different Smad molecules in the epithelium of rat ventral prostate before and after castration, in androgen-sensitive Dunning R3327 PAP prostatic tumor cells from untreated and castrated rats, and after treatment with estrogen. In the ventral prostate, a significant increase of phosphorylated Smad2 (P-Smad2) was observed after castration. In prostatic tumor cells we observed an increased expression of Smad2 and P-Smad2 after treatment. The levels of Smad3 and, in particular, Smad4 were enhanced in the normal ventral prostate, as well as in the tumors after castration. Interestingly, Smad6 and Smad7 expression was also up-regulated in cells with increased Smad2 activation. The staining for Smad2, P-Smad2, Smad3, Smad4, and Smad7 was nuclear in some cells and was present in areas with a large number of apoptotic cells identified by various morphological criteria, formation of apoptotic bodies and, in adjacent sections, by terminal deoxynucleotidyl transferase-mediated nick end labeling assay.

Our results suggest that the signal transduction pathway for TGF-β, leading to apoptosis, is activated in the normal prostate after castration and in the tumor model after castration, without or with estrogen treatment.

INTRODUCTION

Androgen withdrawal by castration is commonly used to treat advanced prostatic carcinoma because the prostate gland is dependent on androgens for its growth and differentiation (1–3). TGF-β1 is a member of a large group of cytokines, including TGF-βs, activins, and BMPs, which are important regulators of cell proliferation, differentiation, apoptosis, and extracellular matrix production of many different cell types (4). In recent years, a mechanism whereby TGF-β transmits its signal from the cell membrane to the nucleus has been elucidated. TGF-β binds to a TβR-II, whereafter another TβR, TβR-I, is recruited into the complex and becomes phosphorylated and activated. The activated TβR-I then propagates the signal through phosphorylation of Smad2 and Smad3, which form heteromeric complexes with each other and with Smad4 that translocate to the nucleus (reviewed in Ref. 5). The Smad genes in mammalian cells are homologues of genes identified in Drosophila and Caenorhabditis elegans, encoding mediators of signaling by TGF-β-like factors in these organisms (6, 7). Certain Smad members (e.g., Smad2 and Smad4) have been found to be deleted or mutated in tumors and could possibly act as tumor suppressor genes (4, 5, 8, 9). Deficiency in Smad3 in mice has been shown to result in advanced and metastatic colorectal cancer (10), whereas compound mutant mice for Smad4 and the APC loci gave rise to an increased formation of intestinal polypsis (11). Furthermore, a subset of juvenile polyposis families carry germ line mutations in Smad4, thus, confirming an important role for Smad4 in the development of gastrointestinal tumors (12). A new subclass of Smads has recently been identified with inhibitory effects on the signal transduction pathway, namely Smad6 (13, 14) and Smad7 (15–17). The mRNA levels of Smad6 and Smad7 increase rapidly after BMP-7 and TGF-β1 stimulation (15, 18), suggesting that they may act in an autocrine feedback loop.

Apoptosis in the prostate, as well as in androgen-dependent prostatic tumors, is regulated by testosterone: aberrations or losses of components in the normal apoptotic pathway could contribute to the development of carcinogenesis of the prostate (2, 19, 20). In normal, as well as in some malignant prostatic carcinoma cells in vitro and in vivo, TGF-β1 has been shown to induce apoptosis. However, the molecular mechanisms for TGF-β-induced apoptosis remain poorly understood (1, 21–24). The Dunning R3327 PAP model is a commonly used and well-accepted model for studies of prostatic tumor biology because it has many similarities to human prostatic carcinoma, such as its androgen sensitivity and morphology (for review see Ref. 25). We have previously reported that castration and, in particular, castration combined with estrogen treatment of animals with the Dunning R3327 PAP tumor cause an increased immunoeexpression of TGF-β1, as well as of TGF-β receptors (22).

In the present study, we have examined the immunoeexpression of Smad proteins and correlated their amount and subcellular localization with apoptosis induced by androgen deprivation, in the normal and malignant prostate.

MATERIALS AND METHODS

Animals and Treatments. Fifteen adult male Sprague Dawley rats were castrated under anesthesia via the scrotal route, and the ventral prostate was dissected out 24 h, 72 h, and 1 week after castration. Five rats were used as controls; they were sacrificed by an overdose of anesthetic, whereafter the ventral prostates were dissected out.

Dunning R3327-PAP prostatic tumors, originally provided by Dr. N. Altman (The Papanicolaou Cancer Research Institute, Miami, FL), were transplanted bilaterally s.c. into the flanks of Copenhagen × Fisher F1 male rats. This tumor subtype is well characterized and androgen sensitive (25). The rats were housed under standard conditions with food and water ad libitum at the animal house in the Department of Physiology, Umeå University, Sweden. Four to 5 months after implantation, when the tumor volume was ∼1300 mm3, the rats were allocated into the following groups: (a) control animals (seven rats with 13 tumors); (b) castrated animals (seven rats with 13 tumors), treated with one single injection of sesame oil and sacrificed after 4 h, 12 h, and 24 h; (c) castrated animals treated with 50 μg of E2 dissolved in sesame oil (eight rats with 15 tumors) and sacrificed after 4 h, 12 h, or 24 h. E2 was purchased from Sigma Chemical Co. (St. Louis, MO). Castration was performed under...
light ether anesthesia via the scrotal route. All animal experiments were done with permission of the ethical committee of Umeå University.

**Antibodies and Immunostaining.** For immunohistochemical analysis of the expression of Smad1 to Smad7, formalin-fixed and paraffin-embedded material was used. The generation and characterization of rabbit antipeptide antisera specific for the Smad1 to Smad5 proteins, as well as methods for the application of the antibodies for immunohistochemistry, have been described in detail (26–28).

Antisera for Smad1 was raised against the peptide TFP (TFPDSFQPN-SHPFHS); for Smad2, against the peptide DQQ (DQQLNQSMDTGSPEAL-SPTT); for Smad3, against the peptide DHQ (DHQMNHSMDAGSPNL-SPNPI); for Smad4, against the peptide HPP (HPPSNRATETYSPALLA); and for Smad5, against the peptide SNN (SNNMIPQTMPSISSRDVQP). An antiserum for Smad6, termed ESP, was raised against the peptide ESPPPPYS-RLSPRDEYKPLD, and for Smad7, antisera termed KAF was raised against the peptide KAFDYEKAYSLQRPNDHE. The peptides were coupled to keyhole limpet hemocyanin (Calbiochem-Boehringer) with glutaraldehyde, mixed with Freund’s adjuvant, and used to immunize rabbits. The specificities of the antisera were tested by subjecting cell lysates from metabolically labeled COS cells transfected with plasmids encoding epitope-tagged Smads to immunoprecipitation with various Smad antisera (27–29). All antisera were specific; however, the Smad1 antiserum made against peptide TFP cross-reacted with Smad8, and the Smad6 antiserum cross-reacted with Smad7 when analyzed in Western blots of COS cell lysates with overexpressed Smads. An antiserum specific for phosphorylated and activated Smad2 (P-Smad2) was raised against the peptide KKKSSp MSp (Sp indicates phosphorylated serine residue) and has been described previously (29, 30). Specificity of the P-Smad2 antibody was tested after immunoprecipitation of metabolically labeled cell lysates from COS cells transfected with Smad2 in the absence or presence of activated TβRI receptors, as well as from various nontransfected cells before and after stimulation with TGF-β1 and BMPs. The P-Smad2 antibody does not recognize nonphosphorylated Smad2 and does not cross-react with other phosphorylated Smads on immunoprecipitation. In Western blots, we observed some cross-reactivity with other P-Smads. Affinity purified antibodies were used at a concentration of 5 μg/ml. The polyclonal antibody was omitted in one section in every experiment, as a negative control. The specificity of the staining was confirmed by blocking experiments using 10–20 times molar excess of the cognate antibody of the corresponding peptide. Immunostaining
Fig. 2. Immunohistochemical expression of Smad2, P-Smad2, Smad3, Smad4, Smad6, and Smad7 in the rat ventral prostate before castration and 24 and 72 h after castration. No major change in the expression level of Smad2 was observed. Note the increased expression of P-Smad2, Smad3, Smad4, Smad6, and Smad7. Some of the epithelial cells show light-microscopical signs of apoptosis in the form of apoptotic bodies (indicated by arrowheads). Endothelial cells (marked with an arrow) stained positive for Smad6 and Smad7. Smad2, Smad3, Smad6, and Smad7 (×400 magnification); P-Smad2 and Smad4 (×300 magnification).
intensity and level of expression was judged using a scale of 0 to 3 (0, negative staining or staining in <25% of the cells; 1, staining in 25–50% of the cells; 2, staining in 50–75% of the cells; 3, intense staining in 75–100% of the epithelial cells).

Detection of Apoptotic Cells. For TUNEL assay, formalin-fixed tumor tissues were used. All chemicals used in the TUNEL assay were obtained from Boehringer Mannheim (Mannheim, Germany), and the assay was performed according to the vendor’s manual. A total of 2000 tumor cells were scored in each tumor section, and the percentage of TUNEL-positive cells was calculated.

Statistics. Values are expressed as means ± SE, unless otherwise indicated. For comparisons between groups, the Mann-Whitney U test was used. A P <0.05 was considered statistically significant.

RESULTS

Apoptosis in Ventral Prostate. Induction of apoptosis in the normal prostate occurs 24–72 h after castration, as confirmed in the present study by TUNEL assay (data not shown). The epithelial cells in the normal prostate undergo massive apoptosis after castration because they are dependent on testosterone for their proliferation and survival. The maximum of apoptosis is reported to occur 3–4 days after castration and to decline thereafter (2, 3, 23).

Apoptosis in the Dunning R3327 PAP Prostatic Tumor. The percentage of apoptotic cells in the intact prostate tumor was 0.6% (± 0.1, n = 6); in the tumors from castrated animals at 24 h after treatment, 1.4% (±0.3, n = 6); and in the tumors from castrated animals, who in addition were treated with estrogen, 1.6% (±0.3, n = 6). Thus, both castration alone and additive treatment with estrogen resulted in a significantly higher percentage of apoptotic cells that were present 24 h after the initiation of treatment. In some areas of the estrogen-treated tumors, massive apoptosis was observed. The growth of Dunning R3327 PAP prostate tumor cells in rats is known to be inhibited by castration and to be further inhibited by additional treatment with estrogen (31). The growth inhibition correlated with an increased number of tumor cells dying by apoptosis, as determined by the TUNEL method (data not shown; See Ref. 22).

Immunostaining for TGF-β1, TGF-β1-type I Receptor, and Cytokeratin. Treatment with estrogens has been shown to lead to an increase in the expression of TβRII, as determined by immunohistochemistry, when compared with the levels in intact and castrated Dunning R3327 PAP tumors (22). We investigated the immunoreactivity of TGF-β1 and TβRI in the normal prostate, as well as in the Dunning tumor, before and after estrogen treatment. We found an increased immunoreactivity of both TGF-β1 and TβRI in the epithelial cells (data not shown), thus, confirming earlier findings (22, 32). By using a cytokeratin antibody (CK5/6), we showed that the apoptotic cells shedded into the lumina were derived from epithelial cells (data not shown).

Expression and Activation of Smad2. Previously, we showed an association of apoptosis in the Dunning model with increased stainings for TGF-β and TGF-β receptors (22). We, therefore, examined the expression of the Smad proteins in the ventral prostate and the Dunning R3327 PAP prostatic tumor by immunohistochemical methods (for overview see Figs. 1-3). Immunostaining for Smad2 in the normal prostate was localized to the cytoplasm of the epithelial cells. After castration, the expression level for Smad2 remained high at all of the different time points studied (24 h, 72 h, and 1 week; Figs. 1 and 2), and staining, to some extent, became more nuclear. In the prostatic epithelial cells, a high expression was found in the cytoplasm of the cells, 72 h and 1 week after castration. To monitor activation of Smad2, an antibody specifically recognizing Smad2, phosphorylated in the COOH-terminal tail by the type I receptor (P-Smad2), was used. The immunohistochemical staining by P-Smad2 was similar to that obtained using an antiserum against Smad2, with granular staining in the ventral prostate and largely confined to nucleus of the cells (Fig. 2). Twenty-four hours after castration, a dramatic and significant increase in P-Smad2 staining was observed in the epithelial cells (P < 0.05; Fig. 2). Some cells showed morphological signs of apoptosis, such as chromatin condensation and nuclear fragmentation. The P-Smad2 staining remained significantly higher in the prostatic epithelial cells also 72 h and 1 week after castration (P < 0.05), when compared with ventral prostate from untreated animals (Fig. 1).

In the Dunning R3327 PAP prostatic tumor, the stainings for Smad2 and P-Smad2 were significantly lower than in the normal ventral prostate (Figs. 1 and 3). However, 24 h after castration, or after castration combined with estrogen treatment, a significant increase of the immunostaining with the P-Smad2 antibody was observed in tumor cells predominantly in the nucleus (Fig. 3; P < 0.05 for both groups).

Expression of Smad3 and Smad4. Immunostaining of Smad3 was seen at a low level, mainly in the cytoplasm of the epithelial cells in the untreated ventral prostate (Figs. 1 and 2). However, a significant increase in the immunoexpression of Smad3 was observed 24 h after castration, particularly in some of the apoptotic epithelial cells (Fig. 2). The immunostaining for Smad3 declined 72 h after castration and remained at a lower level also 1 week after castration, when compared with the level 24 h after castration.

In the prostatic tumor, a significantly lower immunoreactivity level of Smad3 was found, compared with normal prostate (P < 0.05; Figs. 1–3). After castration alone (Figs. 1 and 3) or with additive estrogen treatment (Fig. 1), a significant increase of Smad3 in the nuclei of the tumor cells was seen. Some of these tumor cells showed signs characteristic of early apoptosis (Fig. 3).

Immunostaining of Smad4 in the ventral prostate was found mainly in some of the basal epithelial cells; the staining increased 24 h after castration (P < 0.05; Fig. 2). An intense staining of Smad4 was seen in the cytoplasm of the epithelial cells and in apoptotic bodies shedded into the lumina, where they appeared to have been engulfed by macrophages. Immunoreaction for Smad4 was also seen in some cells with fragmented nuclei, still located in the epithelial compartment. In some areas showing only remnants of fragmented epithelial cells, indicating that a large number of cells had died by apoptosis, an intense immunoreaction for Smad4 was observed. However, the expression level of Smad4 decreased 72 h after castration and remained low 1 week after castration (Figs. 1 and 2).

In the intact Dunning tumor, immunoreexpression of Smad4 was mainly found in endothehial cells, while the expression level in the tumor cells was low (Figs. 1 and 3). After castration, a significant increase of the immunohistochemical staining for Smad4 was observed (Fig. 3). An intense staining for Smad4 was present in the nuclei of some tumor epithelial cells, showing signs of early apoptosis (isolated, irregular, and with chromatin margination).

Fig. 3. Immunohistochemical expression of Smad2, P-Smad2, Smad3, Smad4, Smad6, and Smad7 in the Dunning R3327 PAP prostatic tumor before castration and 24 h after castration, with or without additive E2. Note the low expression level of Smad2 in the tumor cells, which increases after castration. In addition, note the increased expression of P-Smad2, Smad3, Smad4, Smad6, and Smad7 after castration, with or without E2 treatment. Some of the epithelial cells show light-microscopical signs of apoptosis in the form of apoptotic bodies (indicated by arrowheads). Endothelial cells (marked with an arrow) stained positive for Smad4, Smad6, and Smad7. Smad2, Smad3, Smad6, and Smad7 (×400 magnification); P-Smad2 and Smad4 (×300 magnification).
After additive estrogen treatment, an intense staining was observed both in the nuclei and in the cytoplasm of some of the tumor cells (Fig. 3).

**Expression of Smad1 and Smad5.** Smad1 and Smad5 are not activated by TGF-β, but rather by BMPs. The expression level of Smad1 was low in both the normal and malignant prostate and was only slightly increased after castration alone or with additive estrogen treatment (Fig. 1). The immunohistochemical staining for Smad5 in the ventral prostate before and after castration did not show any significant differences (Fig. 1). In the Dunning tumor, a significant increase of Smad5 was observed after castration with additive estrogen treatment (Fig. 1).

**Expression of Smad6 and Smad7.** The immunohistochemical staining for Smad6 increased significantly after castration and remained at a high level also 1 week after castration (Figs. 1 and 2). Both nuclear and cytoplasmic localization of Smad6 were observed in some of the epithelial cells after castration in the ventral prostate and after treatment of the prostatic tumors. In the ventral prostate, some cells in the stromal compartment were also positive for Smad6 and, 1 week after castration, a major part of stromal cells stained positive with Smad6 antibody. In the Dunning tumor, a significant increase of Smad6 was observed after castration. In one tumor sample from a castrated plus estrogen-treated tumor, a high expression of Smad6 was found (Fig. 3). Although Western blot experiments, using cell lysates from transiently Smad6 or Smad7 cDNA-transfected COS cells, revealed that the ESP antiserum against Smad6 has the potential to recognize Smad7, the ESP antiserum seems to specifically recognize Smad6 in immunohistochemistry because, essentially, no overlap in the staining pattern with the specific Smad7 antiserum KAF was observed (see below).

In some areas of the ventral prostate, the basal epithelial cells showed a high immunoreactivity of Smad7 (Fig. 2). Endothelial cells also stained positive for Smad7 in the ventral prostate, as well as in the Dunning R3327 PAP tumor. Twenty-four hours after castration, a slight decrease of Smad7 staining was found, whereas 72 h and 1 week after castration the immunoreactivity of Smad7 significantly increased again (P < 0.05). There was a significantly weaker immunoreactivity of Smad7 in the intact Dunning tumor compared with the intact ventral prostate (P < 0.05). Because Smad7 mRNA levels have been reported to be rapidly induced in vitro after TGF-β stimulation, prostatic tumor tissues were examined also at 4 h and 12 h after castration, and also after additional E₂ treatment. A significantly increased immunostaining for Smad7 was detected at both these time points (P < 0.05; data not shown). Smad7 was localized in the nuclei in some of the tumor cells after estrogen treatment (Fig. 3). In conclusion, some major differences between the staining pattern for Smad6 and Smad7 were found. The basal epithelial cells in the ventral prostate before castration did not show any detectable immunostaining for Smad6, although the immunoreactivity of Smad7 was high in some areas (Fig. 3). Furthermore, the stromal cells, including endothelial cells in the prostate, showed strong staining using the Smad6 antibodies before and after castration, whereas this staining using the Smad7 antibodies was mainly confined to epithelial and endothelial cells.

**DISCUSSION**

Epithelial cells in the normal prostate undergo massive apoptosis on androgen deprivation because testosterone is needed for proliferation, as well as for survival, of the cells (2, 3). Estrogen treatment in combination with castration of rats carrying the Dunning R3327 PAP tumor results in an increased number of tumor cells dying by apoptosis, compared with untreated animals and animals treated by castration alone (22, 31). The molecular mechanisms by which apoptosis is induced in the normal prostate after androgen withdrawal is not well understood. The decrease of androgens leads to a rapid increase of the transcription of several genes, including TGF-β1, that normally are repressed by testosterone in the intact prostate (24, 33, 34). Recently, Smad proteins have been shown to transduce ligand-induced signals from serine/threonine receptors to the nuclei. In the present study, we report that an activation of Smad2 and an up-regulation of the levels of Smad2, Smad3, Smad4, Smad6, and Smad7 occur in the normal and malignant prostate after castration that are associated, in time, with the induction of apoptosis.

TGF-β has been associated with induction of apoptosis both in the normal and malignant prostatic cells in vivo and in vitro (1, 3, 21–23). However, during tumor progression, the inhibitory effect of TGF-β1 on the proliferation of the epithelial cells are lost, and TGF-β1 may, instead, promote tumor growth through its potent immunosuppressive and angiogenic effects (35, 36). In human prostatic tumor tissues, decreased levels for TβR-II have been reported (37), and it has recently been shown that the loss of TβR-I expression in human prostate cancer tissues is associated with a poor prognosis (38). A mutation coding for loss of function has been observed in the TβR-I gene in a TGF-β-sensitive prostatic carcinoma cell line, LNCaP (39). These alterations make prostatic cancer cells insensitive to the increased expression levels of TGF-β seen in advanced prostatic carcinoma (40, 41).

In the hormone-sensitive Dunning R3327 PAP prostatic carcinoma model, we have earlier reported that the expression of TβR-I and TβR-II increased in tumor cells after castration and, even more so, on additive estrogen treatment (22). We observed a strong association between the expression of TGF-β1 and the number of apoptotic cells identified by TUNEL assay, supporting the idea that TGF-β1 is an inducer of apoptosis after androgen withdrawal in this highly differentiated prostatic tumor (22). In the present study, we observe increased levels of expression of Smads involved in TGF-β signal transduction (i.e., Smad2, Smad3, and Smad4) in the epithelial cells of the normal prostate after castration, as well as in the Dunning tumor cells. Moreover, in prostate epithelial cells after castration, we could demonstrate an increased activation of Smad2 detected by an antiserum specifically recognizing receptor-phosphorylated Smad2. Many of the apoptotic cells in the castrated prostate were shedded into the lumina, and some of the Smads expressing cells are likely to be macrophages, engulfing the dying epithelial cells. However, apoptotic cells were still present in the epithelium, identified by their morphology with margination of the fragmented DNA toward the nuclear membrane (2, 42). These cells also showed immunoreactivity for TGF-β-type 1 receptor (data not shown), Smad2, P-Smad2, Smad3, and Smad4 (Figs. 1 and 2). Ectopic expression of Smads has been found to potentiate signaling of TGF-β family members and/or to induce ligand-independent responses (27, 28, 43–46). Furthermore, overexpression of Smad4, in particular together with Smad3, has been shown to induce apoptosis of Madin-Darby canine kidney cells in vitro (47). Thus, the observed up-regulation of Smad3, Smad4, and Smad2 may make cells more susceptible to TGF-β stimulation. Translocation of Smad complexes into the nuclei of prostatic cells in response to TGF-β may activate the transcription of target genes, leading to apoptosis in those cells, but not in the surrounding cells that lack the increased Smad expression. Taken together, these observations support the notion that the epithelial cells undergo apoptosis due to TGF-β-induced signaling through Smad2, Smad3, and Smad4.

The expression levels of inhibitory Smad6 and Smad7 were also
investigated in this study, and the endothelial localization of Smad6 and Smad7 is consistent with previous findings (17).4

Increased expression of Smad6 and Smad7 was seen after castration, and some Smad7-positive cells also showed signs of apoptosis, such as chromatin condensation and formation of apoptotic bodies, as observed for the other Smads. Smad6 and Smad7 may regulate the intensity or duration of the TGF-β signaling response in the prostate. Moreover, we have found that ectopically expressed Smad7 in malignant cells is predominantly localized in the nucleus (49). The function of the inhibitory Smads in the nucleus is currently under investigation.

In the Dunning prostatic carcinoma model, we also noticed an increase of Smad proteins after castration, although it was not as dramatic as in the normal prostatic cells after castration. Interestingly, there was no expression of Smad2 detected by immunohistochemistry in the untreated Dunning tumors. This finding was in sharp contrast to the strong and intense staining for Smad2 in the cytoplasm of the intact ventral prostatic epithelial cells. In tumors growing in castrated rats, treated or not with estrogen, an increased staining for Smad2 was observed in the nuclei, particularly in areas where cells showed early morphological signs of apoptosis. The weaker staining of Smad3 found in the Dunning tumor compared with the normal prostate, correlates with the lower degree of apoptosis induced by castration and/or estrogen treatment.


Increased Smad Expression and Activation Are Associated with Apoptosis in Normal and Malignant Prostate after Castration

Greger Brodin, Peter ten Dijke, Keiko Funa, et al.


Updated version  Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/59/11/2731

Cited articles  This article cites 43 articles, 18 of which you can access for free at:
http://cancerres.aacrjournals.org/content/59/11/2731.full.html#ref-list-1

Citing articles  This article has been cited by 32 HighWire-hosted articles. Access the articles at:
/content/59/11/2731.full.html#related-urls

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.